Expression and functional implications of luteal endothelins in pregnant and non-pregnant dogs

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Abstract: Luteal development is regulated by many locally produced mediators, e.g., prostaglandins and angiogenic factors. However, the role and function of vasoactive factors in the canine corpus luteum (CL) remain largely unknown. Consequently, expression of the endothelin (ET) receptors-A and -B (ETA and ETB, revealing vasoconstriction and vasodilator properties respectively), the ET-converting enzyme (ECE1) and ET1, -2 and -3 were investigated in CL from non-pregnant dogs (days 5, 15, 25, 35, 45 and 65 post-ovulation), and at selected stages of pregnancy (pre-implantation, post-implantation, mid-gestation), and during normal and antigestagen-induced prepartum luteolysis/abortion. The interrelationship between PGE2 and the ET system was investigated in PGE2-treated canine primary lutein cells from early CL. ET1 did not change significantly over time; ET2, ECE1 and ETB were elevated in early CL and were downregulated towards the mid/late-luteal phase. The prepartum increase of ET2 was significant. ET3 increased gradually, and was highest in late CL and/or at prepartum luteolysis. ETA remained constant until the late CL phase and increased only during prepartum luteolysis. ET1 was localized to the luteal cells, and ET2, ET3 and ETA to vascular endothelium. ECE1 and ETB were detected at both locations. Except for upregulated ET1 and lack of effect on ET2, antigestagen applied to mid-pregnant dogs evoked similar changes to those observed during normal luteolysis. PGE2 upregulated ETB in treated cells; ETA and ET1 remained unaffected, and ET2 decreased. A modulatory role of the ETs in canine CL, possibly in association with other factors (e.g., PGE2 and progesterone receptor), is strongly indicated.

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Title: Expression and functional implications of luteal endothelins in pregnant and non-pregnant dogs.

Short title: Luteal endothelin system in the dog.

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Abstract

Luteal development is regulated by many locally produced mediators, *e.g*., prostaglandins and angiogenic factors. However, the role and function of vasoactive factors in the canine corpus luteum (CL) remain largely unknown. Consequently, expression of the endothelin (ET) receptors-A and -B (ETA and ETB,, revealing vasoconstriction and vasodilator properties, respectively), the ET-converting enzyme (ECE-1) and ET-1, -2 and -3, was investigated in CL from non-pregnant dogs (days 5, 15, 25, 35, 45 and 65 post-ovulation), and at selected stages of pregnancy (pre-implantation, post-implantation, mid-gestation), and during normal and antigestagen-induced prepartum luteolysis/abortion. The interrelationship between PGE2 and the ET system was investigated in PGE2-treated canine primary lutein cells from early CL. *ET-1* did not change significantly over time; *ET-2*, *ECE-1* and *ETB* were elevated in early CL and were downregulated towards the mid/late-luteal phase. The prepartum increase of *ET-2* was significant. *ET-3* increased gradually, and was highest in late CL and/or at prepartum luteolysis. *ETA* remained constant until the late CL phase and increased only during prepartum luteolysis. ET-1 was localized to the luteal cells, and *ET-2*, *ET-3* and ETA to vascular endothelium. ECE-1 and ETB were detected at both locations. Except for upregulated *ET-1* and lack of effect on *ET-2,* antigestagen applied to mid-pregnant dogs evoked similar changes to those observed during normal luteolysis. PGE2 upregulated *ETB* in treated cells; *ETA* and *ET-1* remained unaffected, and *ET-2* decreased. A modulatory role of the ETs in canine CL, possibly in association with other factors (*e.g*., PGE2 and progesterone receptor), is strongly indicated.

Key words: domestic dog, corpus luteum (CL), endothelin system.

Introduction

The Corpus luteum (CL) is a highly vascularized, transient endocrine tissue. Its development and maintenance depend on an adequate blood supply (reviewed in (Fraser & Wulff 2003)). Prior to ovulation, the basement membrane of growing ovarian follicles constitutes the borderline between the vascularized and non-vascularized follicular compartments (Suzuki *et al.* 1998, Grazul-Bilska *et al.* 2007, Martelli *et al.* 2009, Kowalewski *et al.* 2015). Its rupture during ovulation is followed by rapid spreading of blood vessels into the follicular antrum and establishment of a dense vascular network that in mature CL provides virtually every lutein cell with access to a capillary. Thus, angiogenesis is an important process needed for rapid luteal formation. It is tightly regulated by numerous locally produced mediators such as angiogenic factors (Reynolds *et al.* 2000, Fraser & Wulff 2003, Zalman *et al.* 2012). Among these, endothelins (ETs) play an important role in regulating many ovarian functions including vascularization, steroidogenesis, ovulation and folliculogenesis, as well as luteal regression and/or luteolysis (Apa *et al.* 1998, Girsh & Dekel 2002, Ko *et al.* 2006, Cacioppo *et al.* 2014). Endothelial-derived ETs consist of three different isoforms, ET-1, -2, and -3, which convey their effects through ETA and ETB receptors (Yanagisawa *et al.* 1988, Nussdorfer *et al.* 1999). Although activation of ETA causes a pronounced vasoconstriction, ETB receptor occupation results in vasodilatation by induction of the nitric oxide pathway (Yanagisawa & Masaki 1989). While ETA has a very high affinity for ET-1 and ET-2, ETB has a similar affinity to all the endothelin types (Nussdorfer *et al.* 1999). ET-2 is the only endothelin that is upregulated in pre-ovulatory follicles at the time of ovulation and, as suggested for humans, rats and mice, its elevated levels might result from the hypoxic condition observed within growing follicles (Ko *et al.* 2006, Na *et al.* 2008, Bridges *et al.* 2010, Choi *et al.* 2011). The functional importance of ET-2 and ETB for ovulation, CL formation and luteal P4 production was shown in mice (Palanisamy

et al. 2006, Cacioppo *et al.* 2014). ETs also influence steroidogenesis through the activation of ETA (Iwai *et al.* 1991, Tedeschi *et al.* 1994, Kamada *et al.* 1995, Girsh *et al.* 1996). ET-1 was shown to inhibit LH-, FSH- and hCG- stimulated P4 secretion by porcine, human and rat granulosa cells, as well as by cultured bovine luteal cells (Iwai *et al.* 1991, Flores *et al.* 1992, Tedeschi *et al.* 1994, Kamada *et al.* 1995, Girsh *et al.* 1996). Furthermore, as shown in cattle (Ohtani *et al.* 1998), ETs seem to be involved in PGF2α-induced luteolysis. Thus, utilizing a luteal microdialysis system and applying PGF2α in cows, a steep rise in ET-1 concentration was detected within the regressing CL as well as in the ovarian venous blood (Ohtani *et al.* 1998). Similarly, PGF2α injections in sheep, cows and rabbits resulted in elevated luteal mRNA expression of *ET-1* (Milvae 2000, Wright *et al.* 2001, Boiti *et al.* 2007). Interestingly, *in vivo* blockage of the ETA receptor mitigated the ET-1-mediated luteolytic action of PGF2α in ewes and cows (Hinckley & Milvae 2001, Watanabe *et al.* 2006).

In contrast to other mammalian species, none of the above information is available for the dog, a species in which the maintenance of pregnancy depends entirely upon the luteal secretion of P4 as its only major source, both in pregnant and non-pregnant cyclic animals.

As in other species, following ovulation and formation of the *corpus hemorrhagicum*, the canine CL is rapidly vascularized (see review in (Kowalewski 2014)). During this time, PGs, especially PGE2, are among the most potent luteotropic factors regulating CL functions (Kowalewski *et al.* 2015). As concluded from the considerably decreased expression of vascular endothelial cell-bound endoglin (Hoffmann *et al.* 2004a, Hoffmann *et al.* 2004b), the vascularization rate slows down by the mid-luteal phase and does not change significantly later on, until the late luteal phase in non-pregnant bitches. From this, it has been concluded that the reduction of vascular network density does not seem to be the major factor regulating luteal regression in dogs (Hoffmann *et al.* 2004a, Hoffmann *et al.* 2004b). Corroborating these results, expression of the

vascular endothelial growth factor (VEGF) system also remained unaffected during prepartum luteolysis (Kowalewski 2014). The initially increased vasculogenic and angiogenic activities are evidenced by the expression of members of the VEGF system in steroidogenic and non-steroidogenic cells (Mariani *et al.* 2006, Papa Pde *et al.* 2013).

In order to fill the existing knowledge gap regarding the potential function of vasoactive factors in canine CL and, thereby, to better understand the para/autocrine regulatory mechanisms governing its function, the present study investigated the expression and localization of ET-1, -2 and -3, and of ETA and ETB, as well as of endothelin converting enzyme (ECE-1; responsible for activating of endothelins) in CL from non-pregnant and pregnant dogs at selected times during the luteal lifespan. Additionally, in view of PGE2 being one of the most important luteotropic factors in the dog, the effects of PGE2 on the ET system were investigated in canine primary lutein cells isolated from early CL.

Materials and Methods

Tissue collection and preservation

Canine luteal samples were collected from experimental animals by routine ovariohysterectomy (OHE) during the non-pregnant luteal phase and pregnancy. The time of ovulation was determined by regular measurements of P4 concentrations in peripheral blood plasma at 2-3 day intervals and by vaginal cytology. The day when P4 levels reached at least 5ng/ml was assigned as the day of ovulation (Concannon *et al.* 1989). Tissue samples were assigned to the following groups:

Non-pregnant dogs: post-ovulation (p.o.) day 5 (n=5), day 15 (n=5), day 25 (n=5), day 35 (n=5), day 45 (n=5) and day 65 (n=5).

Pregnant dogs: pre-implantation (days 8-12, n=5), post-implantation (days 18-25, n=5), mid-gestation (days 35-40, n=5) and prepartum luteolysis (n=3). The day of mating was designated as day 0 and was 2-3 days after ovulation. In the pre-implantation group, pregnancies were determined by the presence of embryos in uterine flushings. Prepartum luteolysis was assessed by regular measurements of circulating P4 concentration at 6h intervals beginning with gestational day 58; OHE was performed when P4 levels dropped below 3ng/ml in two consecutive measurements. Additionally, abortions were induced in mid-pregnant dogs with an antigestagen, Aglepristone (Alizine®, Virbac, Bad Oldesloe, Germany; 10mg/Kg bw, 2x/24 h apart) and OHE was performed 24h (n=5) and 72h (n=5) after the 2nd treatment. All animal procedures were approved by the respective authorities for animal experiments at Justus Liebig University Giessen, Germany (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c/2015c GI, 18,14) and by the Faculty of Veterinary Medicine, University of Ankara, Ankara, Turkey (permit no. Ankara 2006/06).

Tissue collection and preservation for immunohistochemistry (IHC) and non-radioactive *in situ* hybridization (ISH), protein extraction for Western blot analysis and total RNA isolations for PCR reactions were performed as described previously (Kowalewski *et al.* 2010a, Kowalewski *et al.* 2011, Gram *et al.* 2013). Thus, immediately after OHE, CL were trimmed of surrounding tissues and either placed in 10% neutral phosphate-buffered formalin for 24h at +4°C for IHC and ISH, or immersed in RNAlater® (Ambion Biotechnology GmbH, Wiesbaden, Germany) for 24 h at +4°C and stored afterwards at -80°C until total RNA isolation or protein extraction.

Primary luteal cell cultures

Canine primary lutein cells were isolated from CL of clinically healthy bitches (n=15), which underwent routine OHE early in diestrus, 7-14 days after the clinical signs of heat had ceased,

and were used in our previous study determining the role of PGE2 in regulating canine luteal steroidogenic acute regulator (STAR) protein expression and steroidogenesis (Kowalewski *et al.* 2013). The mRNA obtained from that previous study was used in the present investigation to test the impact of PGE2 treatment on the expression of genes encoding for members of the ET system. Briefly, cells were isolated using 0.15% Collagenase (Sigma-Aldrich Chemie GmbH, Buch, CH) and undissociated tissue was removed with a 75μm cell strainer (BD Biosciences, Basel, CH). Following washing steps with PBS, cells were suspended in culture medium (DMEM/F12, pH 7.2-7.4, 10% heat-inactivated FBS, 100U/ml penicillin and 100μg/ml streptomycin, 1% ITS (Insulin-Transferrin-Selenium); all from Chemie Brunschwig, AG, BASEL, CH), seeded into 6-well plates and maintained in a humidified incubator at 37°C under 5% CO_2, until reaching 70-80% confluency. The steroidogenic identity of isolated primary luteal cells was confirmed by immunocytochemical 3βHSD staining and STAR immunoflorescence staining (Kowalewski *et al.* 2013). Only non- trypsinized, "passage 0" cells were used for all experiments. Before treatments, cells were rinsed with pre-warmed sterile PBS, which was then replaced by serum-free medium, and used for stimulations with either 0.5 mM N6, 2-dibutyryladenosine-3, 5-cyclic monophosphate (dbcAMP) or 20μM PGE2 (both from Sigma-Aldrich Chemie GmbH) for 6h. After treatment for 6h, upregulated expression of STAR mRNA and protein, and significantly increased steroidogenic output, were previously observed (Kowalewski *et al.* 2013).

Immunohistochemistry

Immunohistochemical (IHC) staining was based on our previously published protocol (Kowalewski *et al.* 2006a, Kowalewski *et al.* 2006b). Briefly, formalin-fixed paraffin-embedded CL tissues were sectioned at 2µm thickness and transferred onto SuperFrost® microscope slides (Menzel-Glaeser, Braunschweig, Germany), allowed to dry overnight at 37°C, deparaffinized with xylene and rehydrated through a graded ethanol series. Epitopes were retrieved by microwave heating in 10mM citrate buffer pH 6.0. Slides were treated with 0.3% hydrogen peroxide in methanol to remove endogenous peroxidase activity. Then they were overlaid with 10% horse serum, which was used as a blocking serum to reduce non-specific binding, and incubated overnight at +4°C with primary antibodies. The following primary antibodies were used: affinity purified goat polyclonal anti-ETA (sc-21194; dilution 1:50), affinity purified goat polyclonal anti-ETB (sc-21196; dilution 1:100), affinity purified goat polyclonal anti-ECE-1 (sc-27558; dilution 1:300), all from Santa Cruz Biotechnology Inc., CA, USA; and mouse monoclonal anti-ET-1 (E166; dilution 1:400) purchased from Sigma-Aldrich. As a negative control, sections were either incubated while omitting the primary antibody, or with non-immune IgGs of the same species instead of primary antibody (the so-called isotype control) applied at the same protein concentration. Following incubation of slides with biotin-labelled secondary antibodies at 1:100 dilution, signal intensity was amplified using the streptavidin-peroxidase Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). The following secondary antibodies were used: biotinylated horse anti-goat IgG BA-9500, and horse anti-mouse IgG BA2000 (all from Vector Laboratories Inc.). Peroxidase activity was visualized using the Liquid DAB+ substrate kit (Dako Schweiz AG, Baar, Switzerland) and slides were counterstained with hematoxylin.

Protein preparation and Western blot analysis

Only luteal samples from pregnant dogs were available for Western blot analysis. Tissues were disrupted while maintaining the cold chain process throughout all procedures and homogenized in NET-2 lysis buffer (50mM Tris-HCl, pH 7.4, 300mM NaCl, 0.05% NP-40) containing 10µl/ml protease inhibitor cocktail (Sigma-Aldrich). Following protein extraction, samples were centrifuged (10,000 X g for 10 min, at 4°C), dissolved in sample buffer (25 mM Tris-Cl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) and SDS-PAGE was performed using 20 µg of tissue homogenate per lane on 10% SDS-polyacrylamide gel as described previously (Kowalewski *et al.* 2010b, Kowalewski *et al.* 2011, Sprekeler *et al.* 2012, Gram *et al.* 2013). Afterwards, proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories GmbH, Munich, Germany) and incubated overnight at +4^{o}C with primary antibody diluted in PBS containing 2.5% skimmed milk. Due to the limited availability of canine-specific or cross-reacting antibodies suitable for Western blot analysis, experiments were restricted to detecting the ETB receptor. Goat polyclonal affinity-purified anti-human ETB primary antibody (same as for IHC; Santa Cruz Biotechnology) was used at 1:500 dilution. The specificity of the primary antibody was verified by blocking the anti-ETB antibody with the epitope-specific blocking peptide (sc-21196; Santa Cruz Biotechnology). Following incubation of membranes with HRP-conjugated secondary donkey anti-goat IgG sc-2056 antibody (Santa Cruz Biotechnology Inc.; dilution 1:15,000), the Immun-Star™ WesternC™ Chemiluminescent Kit substrate (Bio-Rad) was applied according to the manufacturer's protocols and signals were detected using the ChemiDoc™ XRS+ System and Image Lab (Bio-Rad). For internal loading control PVDF membranes were reblotted with monoclonal mouse anti-β-ACTIN antibody (sc-69879; Santa Cruz Biotechnology, dilution 1:1000); secondary HRP goat anti-mouse IgG (W402B) from Promega, Dübendorf, CH, was used at a dilution of 1: 15,000. ImageJ

212 software was applied for assessing the density of bands. Parametric one-way analysis of variance
213 (ANOVA) followed by the Tukey-Kramer multiple comparison test was used to determine the
214 effect of time on luteal ETB protein expression. The GraphPad 3.06 program (GraphPad
215 Statistical Software, San Diego, CA, USA) was applied. Numerical data are presented as the
216 mean ± standard deviation (SD). Representative Western blots are shown.
217
218 **Total RNA isolation and Real Time (TaqMan) PCR**
219 Total mRNA was obtained from CL of pregnant and non-pregnant dogs using TRIzol® reagent
220 following the manufacturer's directions (Invitrogen, Carlsbad, CA, USA). Concentrations of
221 isolated RNA were measured using a NanoDrop 2000C® spectrophotometer (Thermo Fisher
222 Scientific AG, Reinach, CH). DNAse treatment and reverse transcription (RT) were performed
223 following our previously published protocol (Kowalewski *et al.* 2006b, Kowalewski *et al.* 2011).
224 Then, expression of mRNA in luteal samples was determined by semi-quantitative real time
225 (TaqMan) PCR using an automated fluorometer (ABI PRISM 7500 Sequence Detection System,
226 Applied Biosystems, Foster City, CA, USA). The list of primers and TaqMan probes obtained
227 from Microsynth (Balgach, CH) together with amplicon sizes are presented in Table 1. The
228 following commercially available TaqMan Gene Expression Assays® from Applied Biosystems
229 were used: *ET-1* (Prod. No. Cf02622421_m1), *ET-2* (Prod. No. Cf02622240_m1), ET-3 (Prod.
230 No. Cf02622419- g1), *ECE-1* (Prod. No. Cf02627515_m1) and *CYCLOPHILIN A* (Prod. No.
231 Cf03986523-gH). In order to exclude genomic DNA contamination, the so-called "RT-minus
232 controls" were run (*i.e.*, samples where the RT reaction was omitted). Additional controls
233 consisted of using autoclaved water instead of cDNA. The reaction mixture for all samples was
234 prepared as follows: 200nM TaqMan Probe, 300 nM of each primer, 12.5 µl Fast Start Universal
235 Probe Master (ROX)® (Roche Diagnostics, Mannheim, Germany) and 5µl cDNA corresponding

to 100ng total RNA per sample. All samples were run in duplicates. The thermal cycler steps were set as follows: denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 1 cycle at 60°C for 60 sec. Efficiency of each target gene was calculated using the CT slope method and the relative gene expression was calculated using a comparative CT method (ΔΔCT method) according to the manufacturer's protocols for the ABI PRISM® 7500 Sequence Detection System (Applied Biosystems) and as described previously (Kowalewski *et al.* 2006b, Kowalewski *et al.* 2011), and they were normalized using GAPDH, 18SrRNA and cyclophilin. Selected amplicons of each gene were sent for commercial sequencing (Microsynth).

The effects of observational group on expression of target genes in luteal samples were tested by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. For samples obtained from induced abortions, Dunnett's multiple comparison test was performed; the results are presented as n-fold change in target expression compared to the expression at mid-gestation, which served as a non-treated control. In order to test for the effects of treatment with PGE2 on ET system expression in cell cultures, an unpaired two-tailed Student's t-test was performed; numerical data are presented either as Xg ± DF or mean ± SD. All statistical tests were performed with GraphPad 3.06 software. Numerical data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

In situ hybridization (ISH)

Due to the lack of canine-specific antibodies available for ET-2 and ET-3, non-radioactive *in-situ* hybridization was used to localize their expression at the level of transcripts. Formalin-fixed, paraffin-embedded tissues were used and all procedures were performed following our previously published protocol (Kowalewski *et al.* 2006a). Primers used for generating cDNA templates for cRNA synthesis were as follows: *ET-2* forward: 5'-ACA TCA TCT GGG TGA ACA CT-3', *ET-*

2 reverse: 5'-CCT AGG AAA GCG GAT CTT-3' (amplicon length 262bp), *ET-3* forward: 5'-CTA TTG CCA CCT GGA CAT CA -3', *ET-3* reverse: 5'-GCT GGC TCT TCC TCT TTG TC-3' (amplicon length 258bp). The PCR products were visualized using 2% ethidium bromide-stained agarose gel electrophoresis, isolated using the Qiaex II gel extraction system (Qiagen GmbH, Hilden, Germany) and subcloned into the pGEM-T vector (Promega). Upon linearization of plasmids with either NcoI or NotI restriction enzymes (New England Biolabs, Frankfurt, Germany), cRNA probes were synthesized by *in vitro* transcription with a DIG-RNA labelling kit (Roche Diagnostics). Plasmids were sent for commercial sequencing (Microsynth). Following incubation at 37^{o}C overnight, the (DIG) digoxygenin-labeled cRNA probes were detected using alkaline phosphatase-conjugated sheep anti-DIG Fab Fragments (Roche Diagnostics International) diluted 1:5000. The signals were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics International) substrate.

Results

Semi-quantitative assessment of gene expression.

The expression of all target genes was detectable in CL of pregnant and non-pregnant animals at every luteal stage examined. Whereas the expression of *ET-1* mRNA did not change significantly over time, both in non-pregnant and pregnant animals (P=0.91 and P=0.12, respectively; Fig 1A, B), the expression of *ET-2* and *ET-3* was significantly modulated in both situations showing time-dependent effects, which differed at the end of the luteal lifespan (P=0.01 and P<0.0001 for ET2, and P<0.0001 and P=0.0003 for *ET-3*, in non-pregnant and pregnant bitches, respectively). Thus, the expression of *ET-2* mRNA was upregulated during the early luteal phase in pregnant and non-pregnant animals and suppressed significantly by day 35 after ovulation in non-pregnant bitches compared with its highest expression at day 15 (P<0.05). In pregnant dogs, *ET-2* mRNA

decreased significantly ($P<0.05$) following implantation. While its expression remained low in the absence of pregnancy until the end of the observation period (day 65 p.o.) (Fig. 1C), a new and significant increase ($P<0.001$) was noted in pregnant animals at the time of prepartum luteolysis (Fig. 1D). *ET-3* mRNA was lowest in early CL, increased gradually with the development of the CL until the mid-luteal phase, and was significantly elevated during the late luteal stage in non-pregnant animals ($P<0.01$ for day 45 *vs.* day 65 p.o.), or during prepartum luteolysis in pregnant ones ($P<0.05$) (Fig. 1E,F). As for ET receptors, *ETA* was expressed constantly in CL collected from non-pregnant females and did not change significantly throughout the luteal phase ($P=0.91$) (Fig. 2A). This was in contrast to the situation observed in pregnant bitches, in which a time-dependent effect was observed ($P=0.04$); prepartum luteolysis was associated with strongly upregulated *ETA* mRNA expression ($P<0.05$) compared with the post-implantation stage of pregnancy (Fig. 2B). *ETB* mRNA was significantly altered over time ($P=0.0001$) in both situations, *i.e.*, during gestation and in non-pregnant cyclic dogs. It was strongly elevated in early CL, followed by a significant decrease, which in non-pregnant animals was noted at day 25 after ovulation ($P<0.001$) compared with its highest expression at day 15 p.o.. A further gradual decrease was observed towards day 65 p.o. ($P<0.01$ compared with day 15 p.o.) (Fig. 2C). In pregnant bitches, *ETB* decreased significantly at mid-gestation ($P<0.01$) compared with the pre-implantation stage of pregnancy, and remained low at prepartum luteolysis (Fig. 2D). Similarly, at the protein level *ETB* expression was modulated over time ($P<0.0001$), being highest in early CL, and exhibited significantly diminished levels ($P<0.001$) at post-implantation and a further decrease ($P<0.001$) at prepartum luteolysis (Fig. 3B). An expression pattern resembling that of *ETB* was observed for *ECE-1* (Fig. 2E,F). Also here, the expression was time-dependent ($P=0.0005$ and $P=0.0008$ for pregnant and non pregnant females, respectively), with the highest mRNA abundance in early CL (Fig. 2E,F). While its expression

had already decreased strongly ($P<0.01$) by day 15 p.o. in non-pregnant dogs, in pregnant animals the *ECE-1* levels were significantly suppressed ($P<0.001$) during prepartum luteolysis, compared with the highest levels noted at the pre-partum stage of pregnancy (Fig. 2E,F).

The effect of treatment with the antigestagen Aglepristone® on luteal ET system expression was investigated in mid-pregnant dogs (40-45 days of gestation). Non-treated mid-pregnant dogs were used as negative controls. Besides the elevated ($P<0.05$) expression of *ET-1* (Fig. 4A) and unaffected ($P>0.05$) expression of *ET-2* (Fig. 4B), the treatment resulted in similar changes to those observed during normal prepartum luteolysis, *i.e.*, *ET-3* and *ETA* increased significantly 24h after the 2nd treatment ($P<0.05$ and $P<0.01$, respectively), while the expression of *ETB* remained unchanged ($P>0.05$) 24h after the 2nd treatment but decreased significantly at 72h ($P<0.01$) and *ECE-1* remained unaffected ($P>0.05$) (Fig. 4C-E).

Stimulation of canine lutein cells with 20µM PGE2 over a 6h time course did not affect the expression of *ETA*, *ET-1* and *ECE-1* ($P>0.05$) (Fig. 5A,C,E). Whereas *ET-2* was significantly decreased ($P<0.01$) (Fig. 5D), the expression of *ETB* increased significantly ($P<0.05$) (Fig. 5B). In contrast, *ET-3* mRNA expression remained below the detection limit.

Luteal localization of canine ET system expression.

Immunohistochemistry was applied to localize the expression of ET-1, ECE-1, ETA and ETB at the protein level. Due to the lack of canine-specific anti-ET-2 and anti-ET-3 antibodies, their expression was assessed by ISH. ET-1 was predominantly targeted to the luteal cells without marked changes in signal intensity over time in both situations, *i.e.*, in pregnant and non-pregnant animals (Fig. 6). As for *ET-2*, its mRNA was predominantly found in capillaries (Fig 7A), while that of *ET-3* was localized in endothelial cells of small and larger luteal vessels (Fig 7B). As for

ECE-1, signals were detectable throughout the luteal phase and strong immune signals were localized to the vascular endothelial cells, and weaker expression was detected within the luteal cells (Fig 8). The luteal signals tended to be stronger in early diestrus of pregnant and non-pregnant cyclic animals. No or only weak signals were observed in CL for ETA throughout the luteal lifespan but became clearly visible during prepartum luteolysis in pregnant females (Fig. 9B) where its localization was targeted to the endothelial cells of capillaries. In contrast, ETB was clearly detectable and localized in luteal cells, the tunica media of arteries in early CL and some of the interstitial cells (Fig 10). With the progression of the luteal phase, signal intensity decreased (Fig. 10) and the weakest staining was observed in capillaries and luteal cells during late diestrus and at the prepartum luteolysis in pregnant bitches (Fig 10).

Discussion

In this project we evaluated the expression and localization of the members of the ET system in luteal samples from non-pregnant dogs, as well as in CL collected at selected stages of pregnancy, *i.e.*, pre-implantation, post-implantation and mid-gestation, and during normal and antigestagen-induced prepartum luteolysis/abortion.

Whereas the expression of *ET-1*, which was abundantly present in luteal cells, did not change significantly throughout the luteal phase, the early luteal phase of both pregnant and non-pregnant cyclic bitches was characterized by markedly increased levels of vascular *ET-2*. This was associated with concomitantly increased expression of *ECE-1*, which is responsible for enzymatic activation of ETs. A similar expression pattern of ET-2 was observed in CL of cows (Klipper *et al.* 2010), implying a role of this peptide during luteal formation and angiogenesis. The physiological significance of ET-2 in ovarian function was recently demonstrated in knockout mice, in which loss of ET-2 expression resulted in impaired ovulation and luteal

355 formation (Cacioppo *et al.* 2014). Importantly, ET-2 injection into ovaries of these animals
356 augmented ovulation and increased expression of the cytochrome P450 side chain cleavage
357 enzyme (P450scc, CYP11A1) (Cacioppo *et al.* 2014). Similar effects were observed following
358 blockage of the ETB receptor in mice, which also resulted in a decrease and/or delay in follicular
359 rupture (Palanisamy *et al.* 2006). In the dog, as presented herein, the early luteal development
360 was associated with strongly increased expression of *ETB*. Interestingly, its localization seemed
361 ubiquitous, being targeted to both lutein cells and blood vessels, suggesting a possible functional
362 interplay between different structural compartments of the CL with the involvement of ET-
363 dependent pathways. The ETB-mediated proliferative and migratory effects of ETs, *e.g.*, ET-1
364 and ET-3, on endothelial cells and smooth muscles of the vascular tunica media, were shown
365 previously (Alberts *et al.* 1994, Morbidelli *et al.* 1995). Additionally, by activating the nitric
366 oxide pathway in endothelial cells, ETB is capable of increasing vascular permeability and
367 inducing vasodilation (Tsukahara *et al.* 1994). Therefore, ETs exhibit both angiogenic and
368 vasoactive properties. The latter, *i.e.*, vasodilatory and proliferative capabilities of ETB, could
369 also apply to early canine CL, which, following ovulation, requires an increased blood supply to
370 facilitate its rapid growth and enhanced functional steroidogenic performance. During this time,
371 PGs are among the most important regulators of canine luteal function (Kowalewski *et al.* 2013,
372 Kowalewski 2014). Only recently, a functional causality between local PG production and the
373 steroidogenic activity of canine CL was shown (Kowalewski *et al.* 2015). Thus, blocking luteal
374 COX2 (PTGS2) function with a selective blocker, firocoxib (Previcox®, Merial Ltd.), resulted in
375 suppression of luteal PGE2 synthesis and impairment of STAR expression (Kowalewski *et al.*
376 2015). Moreover, the expression of prolactin receptor (PRLR) was diminished in bitches treated
377 with this COX2 blocker. On the other hand, in canine luteal cells isolated from early diestrous
378 bitches, PGE2 stimulated PRLR expression, further implying a possible functional relationship

379 between these two regulatory factors (Kowalewski *et al.* 2015). This seems to be an important
380 observation in view of the role of PRL as the predominant luteotropic factor in dogs during the
381 second half of diestrus (Concannon *et al.* 1987, Okkens *et al.* 1990). In line with this, in the
382 present study, stimulation of canine primary lutein cells with PGE2 significantly upregulated
383 their *ETB* mRNA expression. Therefore, besides being directly involved in the regulation of
384 canine CL function by stimulating STAR expression and increasing P4 output, PGE2 appears to
385 participate indirectly in maintenance of canine luteal function by enhancing the expression of
386 PRLR and ETB.
387 As observed in the present study, the mid and late luteal phases were characterized by decreasing
388 *ETB* and *ET-2* expression. This was associated with concomitantly increasing levels of *ET-3*,
389 which together with *ET-2* was predominantly localized in vascular compartments of the CL.
390 Contrasting with ETB, the potent vasoconstrictor ETA was rather weakly and stably expressed
391 throughout the luteal phase, while its expression increased significantly along with elevated *ET-2*
392 levels during normal prepartum luteolysis. Interestingly, however, for both factors, *i.e.*, *ETA* and
393 *ET-2*, this was not the case during late luteal regression in non-pregnant animals when their
394 expression remained unchanged.
395 In the dog, P4 is considered as a luteotropic factor, so that interfering with its function, *e.g.*, by
396 applying an antigestagen, activates the uterine and placental prostaglandin system and induces a
397 luteolytic cascade leading to pre-term parturition/abortion (Kowalewski *et al.* 2009, Kowalewski
398 *et al.* 2010a). Accordingly, except for the upregulated *ET-1* and unaffected *ET-2* expression,
399 application of an antigestagen on days 40-45 of gestation, which is a time point distant from
400 physiological parturition, evoked similar changes to those observed during prepartum luteolysis.
401 These changes refer especially to the upregulated expression of *ETA*, indicating the involvement
402 of P4 signaling in this process. Interestingly, as shown in this study, while the expression of the

vasoactive ET system reveals strong spatio-temporal changes at the end of canine pregnancy, the angiogenic VEGF-system does not change significantly during normal and induced parturition/abortion (Kowalewski 2014). Therefore, cumulatively, it seems plausible that in contrast to non-pregnant dogs, vascular functionality strongly contribute to structural and functional luteolysis in dogs at term, likely due to the PGF2α-induced and ET-mediated vasoconstrictor properties of ETA. The prolonged, slow process of luteal regression observed in non-pregnant animals remains primarily a passive one, at least where vascular activity is concerned.

For conclusion, the role of the ET system during the cessation of luteal function seems to diverge between pregnant and non-pregnant dogs, i.e., being actively regulated, it possibly contributes to the prepartum luteolysis. Its function also differs from the role of the VEGF system, which remains unaffected in both situations (Kowalewski 2014). The role of PGE2 as an important luteotropic factor has been further strengthened by results obtained from our *in vitro* experiments with canine lutein cell cultures, in which PGE2 was found to be a positive regulator of *ETB* expression.

Conflict of interest: The authors declare that there is no conflict of interest. All authors read and approved the final version of the manuscript.

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Authors contributions: AG, MPK: Idea of the study, experimental design, interpretation of data and writing of the manuscript. SL: involved in the laboratory part of the project, tissue processing. AB, BH: knowledge transfer, critical discussion of the data, editing of the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Figure 1.

Time-dependent expression of canine endothelins (ET) -1, -2 and -3 as determined by Real Time (TaqMan) PCR (mean ± SD). (A, C, E) CL of non-pregnant dogs (days (d) 5-65 post-ovulation), and (B, D, F) CL of pregnant dogs. Bars with different letters differ at $P<0.05$.

Figure 2.

Expression of endothelin receptors -A and -B (ETA and ETB) and endothelin converting enzyme 1 (ECE-1) as determined by Real Time (TaqMan) PCR (mean ± SD) in the (A, C, E) CL from non-pregnant dogs (days (d) 5-65 post ovulation), and (B, D, F) CL collected at selected stages of pregnancy and during prepartum luteolysis. Bars with different letters differ either at $P<0.05$ in B, or at $P< 0.01$ in D and E, or $P<0.001$ in F.

Figure 3.

The expression of endothelin receptor B (ETB) (approx. 50 kDa) was determined by western blot analysis. Representative immunoblots are shown. (A) The epitope-blocking peptide was used to quench the ETB-specific signal in a luteal tissue homogenate derived from pre-implantation CL. (B) Expression profile of ETB protein in CL collected at selected stages of pregnancy and during prepartum luteolysis; 20µg protein was used for each sample. Membranes were re-blotted with β-ACTIN (45 kDa), which served as an internal loading control. Lower panel in (B) represents densitometric values (standardized optical density; SOD) for ETB expression normalized against β-ACTIN. All numerical data are presented as the means ± SD. Bars with different letters differ at $P<0.001$. M=molecular weight marker.

Figure 4.

Expression of endothelin system members (ET-1, -2, -3, ETA, ETB and ECE-1) as determined by Real Time (TaqMan) PCR (mean ± SD) during antigestagen (Aglepristone®)-induced luteolysis/abortion compared with the mid-gestation group used as a non-treated control. Bars with different asterisks differ at $P<0.05$.

Figure 5.

Effect of PGE2 on expression of endothelin system members in canine primary luteal cells collected from non-pregnant dogs during the early CL phase. Cells were cultured in serum-free DMEM/F12 medium with 20μM PGE2. Non-stimulated cells served as a negative control. (A-E) endothelin receptors -A and -B (ETA and ETB) and endothelins (ET) -1 and -2 mRNA expression as determined by real-time (TaqMan) PCR normalized against cyclophilin A, GAPDH and 18SrRNA.

Figure 6.

Immunohistochemical localization of ET-1 in the canine CL at selected time points of pregnancy and on days 5 and 65 post-ovulation in non-pregnant animals. (A) pre-implantation stage, (B) post-implantation, (C) mid-gestation, (D) prepartum luteolysis, and on days (E) 15 and (F) 65 post-ovulation in non-pregnant animals. (A-F) ET-1 is localized to the luteal cells (open arrows) throughout the luteal phase. There is no background staining in the isotype control (insert to F).

Figure 7.

Localization of ET-2 and ET-3 mRNA in the canine CL during prepartum luteolysis by *in situ* hybridization (ISH). (A) ET-2 mRNA is localized to the capillary endothelial cells (solid arrowheads). (B) ET-3 mRNA is localized in vascular endothelial cells (solid arrowheads) and luteal cells (open arrows). There is no background staining in the negative controls (insert to A and B).

Figure 8.

Immunohistochemical localization of endothelin converting enzyme 1 (ECE-1) in the CL of pregnant dogs at selected periods of pregnancy and on days 5 and 15 post-ovulation in non-pregnant animals. (A) Pre-implantation stage, (B) post-implantation, (C) mid-gestation (D) prepartum luteolysis, and on days (E) 5 and (F) 15 post-ovulation in non-pregnant animals. (A-F) ECE-1 expression is localized in luteal cells (open arrows) and vascular endothelial cells (solid arrowheads). There is no background staining in the isotype control (insert to F)

Figure 9.

Immunohistochemical localization of endothelin receptors A (ETA) in CL of pregnant dogs at selected time points of pregnancy, and on days 5 and 65 post-ovulation in non-pregnant animals. (A) Pre-implantation stage, (B) during prepartum luteolysis, and on days (C) 5 and (D) 65 post-ovulation in non-pregnant animals. (A) No or only weak signals were observed during pregnancy in luteal tissue (pre-implantation stage shown). (B) Strong signals were localized in the capillary endothelial cells (solid arrows) during prepartum luteolysis. The capillary endothelial cell expression of ETA is shown at higher magnification in the insert to B. There was no or only

weak staining throughout the non-pregnant diestrus (shown at days 5 and 65 post-ovulation). There is no background staining in the isotype control (insert to D).

Figure 10.

Immunohistochemical localization of endothelin receptors B (ETB) in the CL of pregnant dogs during pregnancy and on days 5 and 65 post-ovulation in non-pregnant bitches. (A) pre-implantation stage, (B) post-implantation, (C) mid-gestation (D) prepartum luteolysis, and on days (E) 5 and (F) 65 post-ovulation in non-pregnant bitches. (A) During pre-implantation, luteal ETB expression is localized to the luteal cells (open arrows), tunica media of arteries (solid arrowheads) and interstitial cells (open arrowheads). (B) At post-implantation and (C) mid-gestation, luteal signals are localized in luteal cells (open arrows) and interstitial cells (open arrowheads in B). (D) During prepartum luteolysis, signals are localized in luteal cells (open arrows) and capillary pericytes (solid arrows). During day (E) 5 post-ovulation in non-pregnant animals, ETB expression is localized to the luteal cells (open arrows) and tunica media of arteries (solid arrowhead). At (F) day 65 post-ovulation in non-pregnant animals, signals are localized in luteal cells (open arrows) and capillary pericytes (solid arrow). There is no background staining in the isotype control (insert to F).

Table1

List of primers and TaqMan Probes used for semi-quantitative RT-PCR

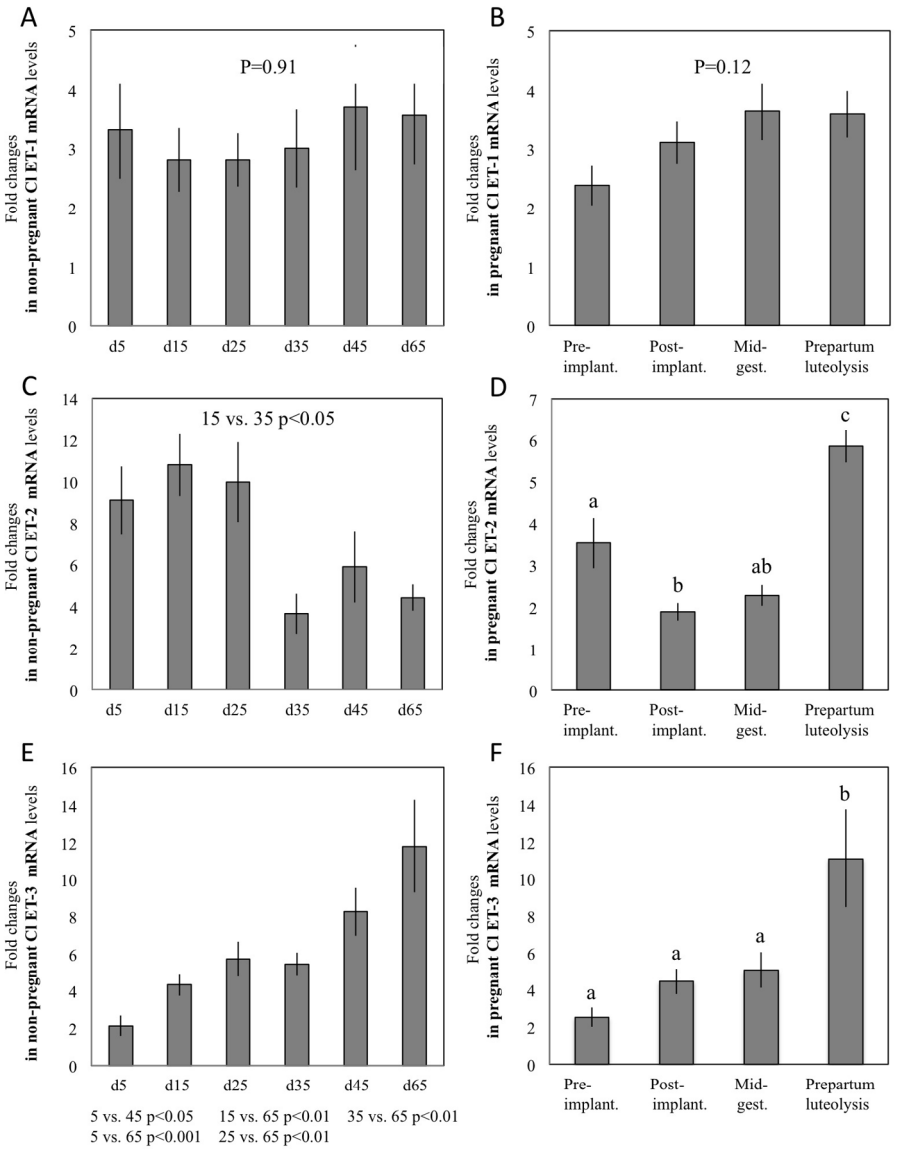

Figure 1

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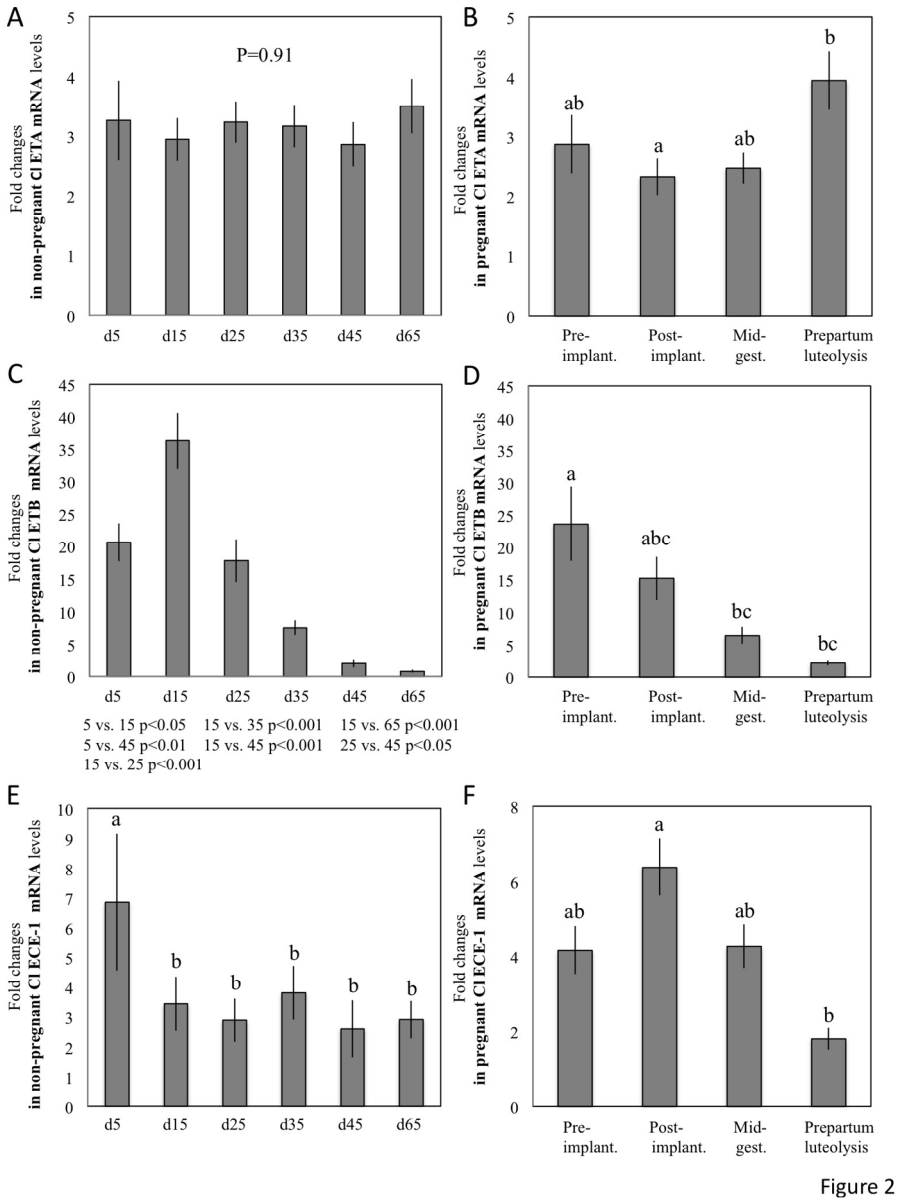

Figure 2

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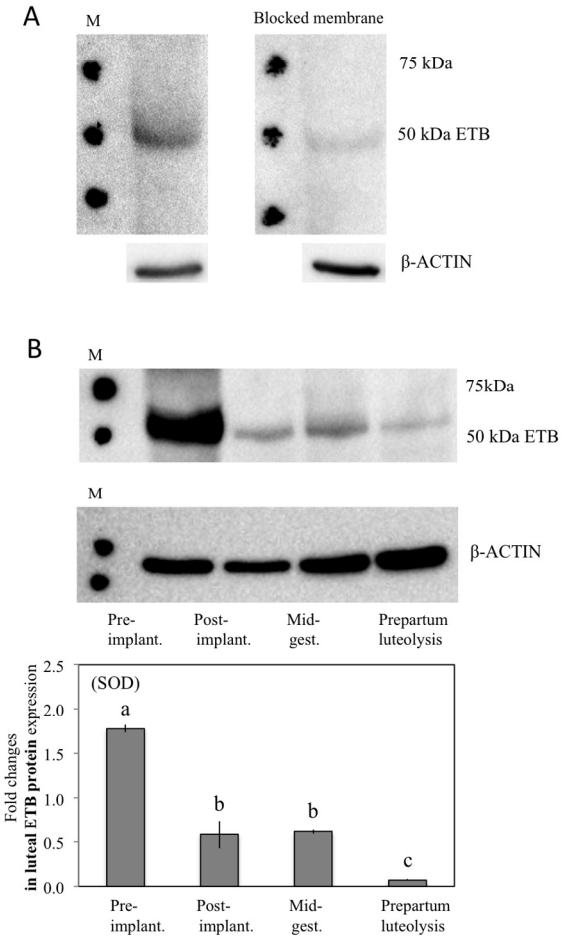

Figure 3

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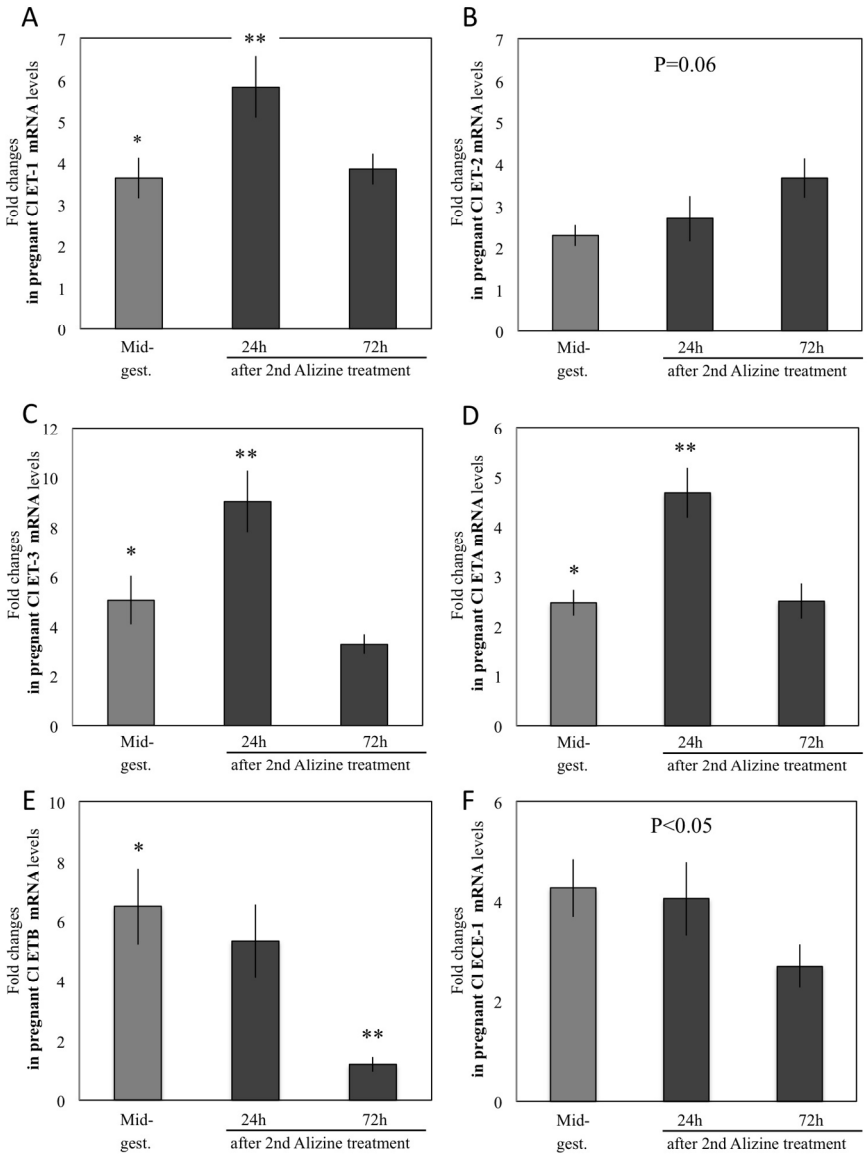

Figure 4

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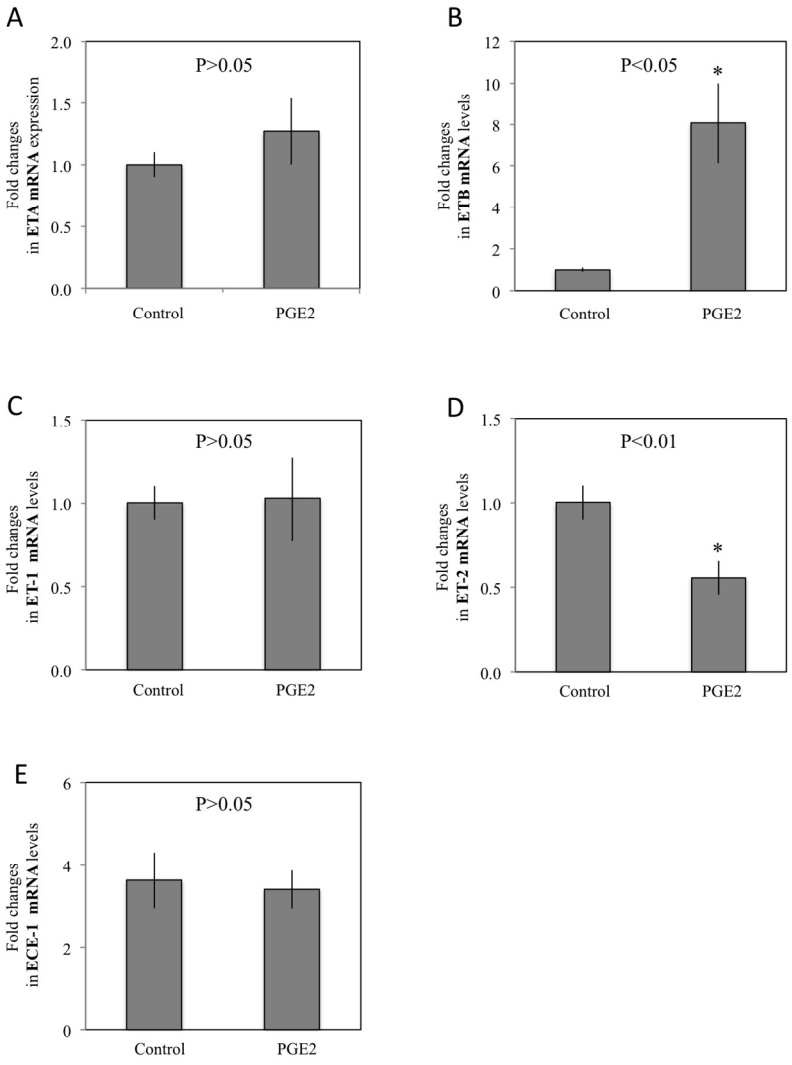

Figure 5

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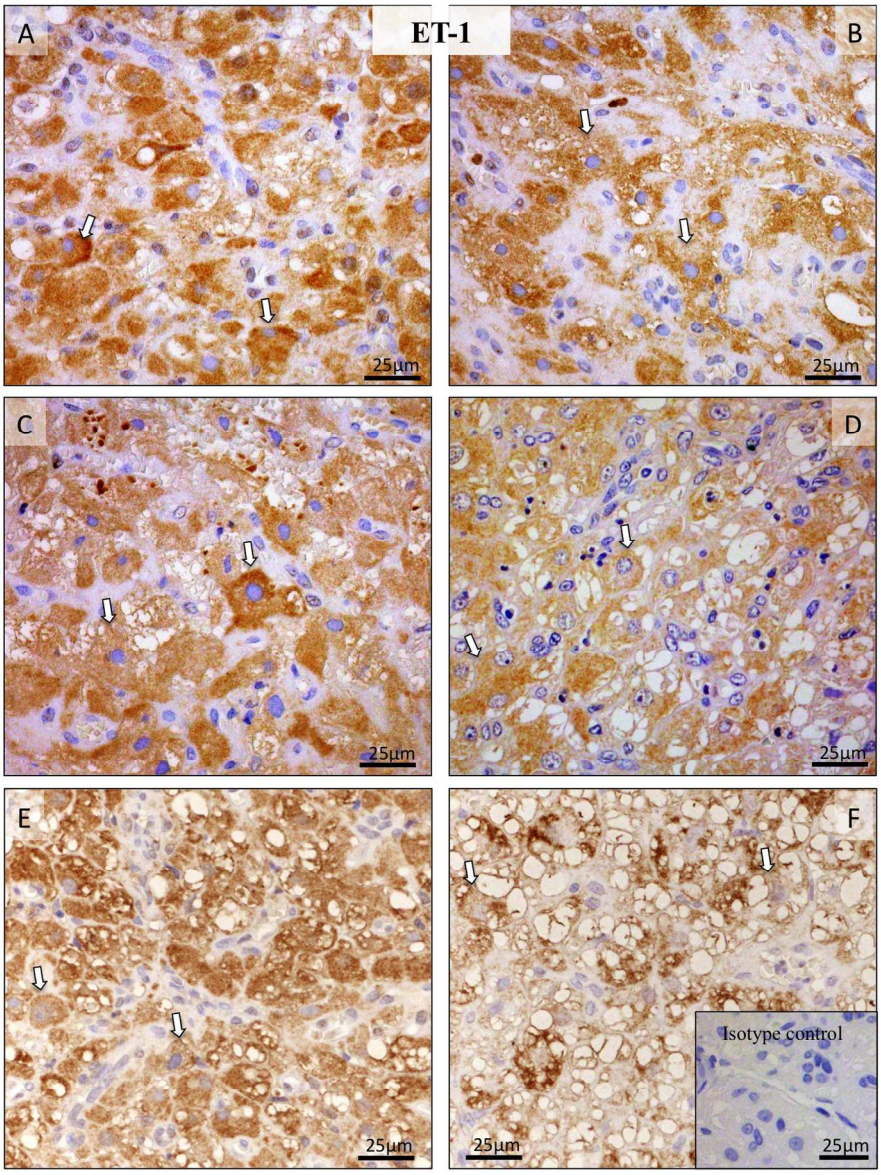

Figure 6

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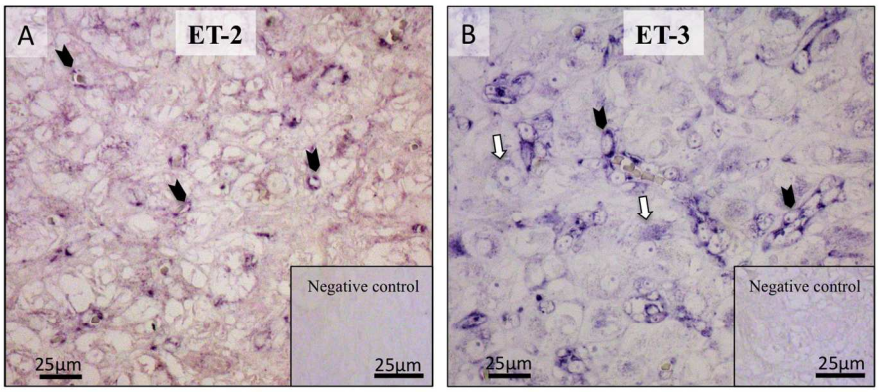

Figure 7

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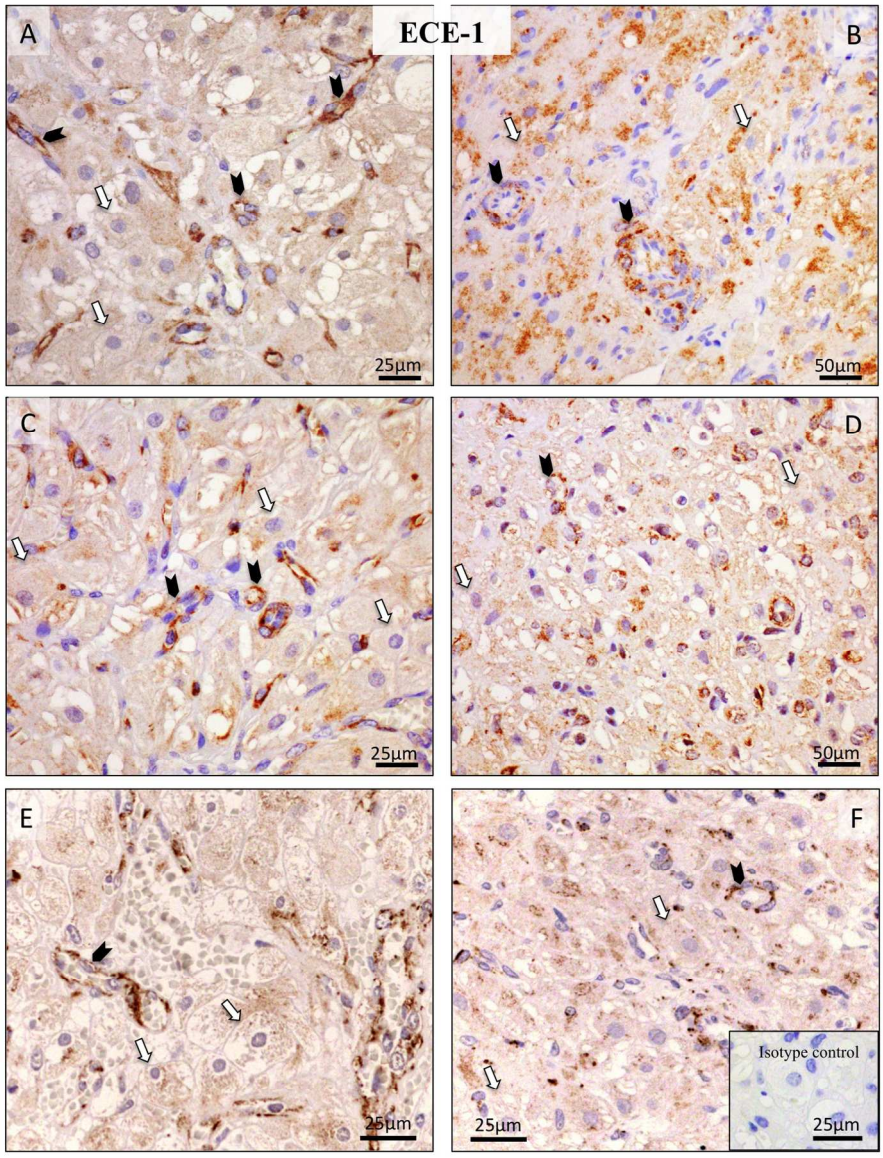

Figure 8

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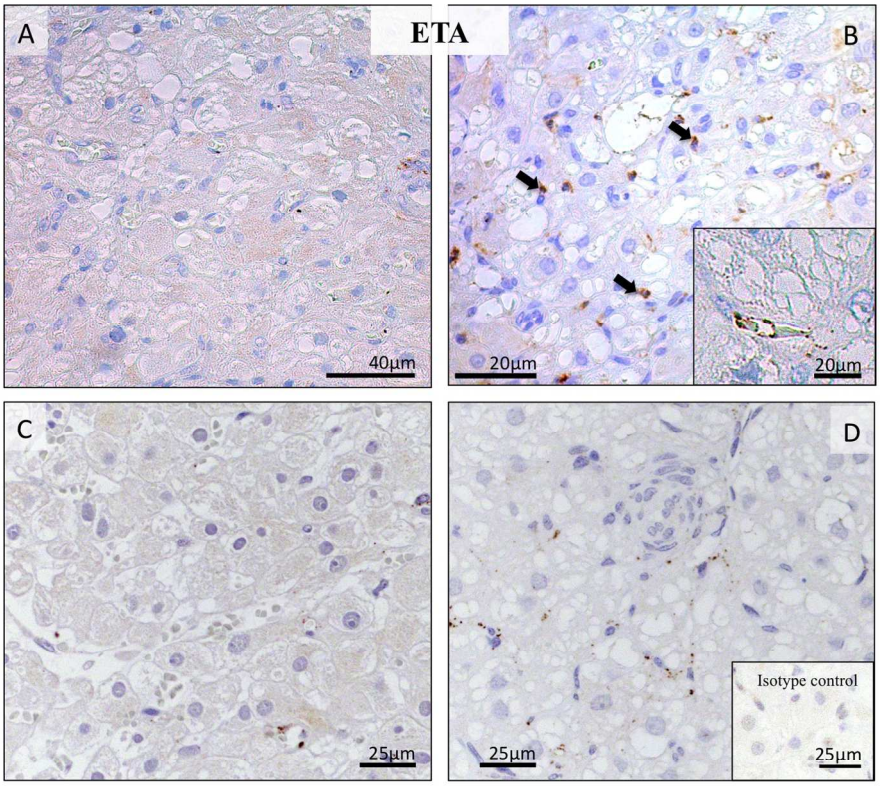

Figure 9

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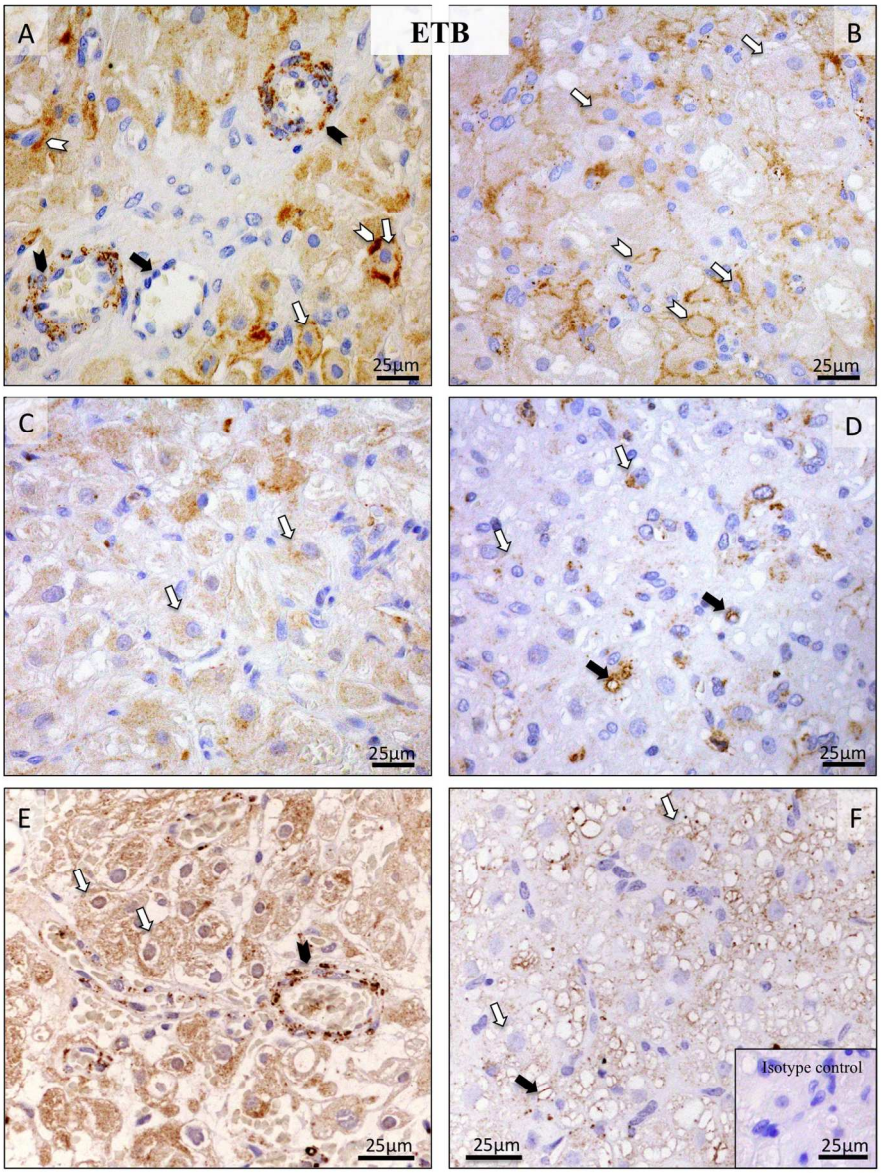

Figure 10

549x732mm (72 x 72 DPI)

Primer	Accession Numbers	Primer Sequences	Product Length
GAPDH	AB028142	Forward: 5’-GCT GCC AAA TAT GAC GAC ATC A-3’ Reverse: 5’-GTA GCC CAG GAT GCC TTT GAG-3’ TaqMan probe: 5’-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3’	75bp
18SrRNA	FJ797658	Forward: 5’-GTC GCT CGC TCC TCT CCT ACT-3’ Reverse: 5’-GGC TGA CCG GGT TGG TTT-3’ TaqMan probe: 5’-ACA TGC CGA CGG GCG CTG AC-3’	125bp
ETA	NM_001031632.1	Forward:5’-GGC CCCAAC GCA CTG ATA-3’ Reverse:5’-CCC GCC AGA AGC TTA AAC AC-3’ TaqMan probe:5’-CCA GCC TTG CCC TTG GAG ACC TTA TC-3’	92bp
ETB	NM_001010943.2	Forward: 5’-CAT CAT CGG GAA CTC CAC ACT-3’ Reverse:5’-CAG AGC CAG GCT GGC TAT CA-3’ TaqMan probe:5’-CAA GAA CAA GTG CAT GCG AAA CGG C-3’	91bp